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Bacterial challenge and eicosanoids act in plasmatocyte spreading

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Abstract

We report on experiments designed to more thoroughly document the roles of eicosanoids as crucial elements in cell spreading and on experiments designed to test the hypothesis that in vivo bacterial infections influence cell spreading on glass surfaces. We used hemocytes prepared from tobacco hornworms, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) and four species of bacteria (*Serratia marcescens*, *Escherichia coli*, *Bacillus subtilis*, and *Micrococcus luteus*) in each experiment. Our protocols yielded several important points: (i) hemocytes prepared from hornworms at 15 and 60 min following infection with, separately, each of the four bacterial species were fundamentally altered in size (all less than the 15-μm counting cut-off) and none of the hemocytes exhibited cell-spreading behavior; (ii) the influence of bacterial challenge on cell spreading declined with incubation time post-challenge; (iii) conditioned medium (CM) prepared by exposing hemocytes to bacterial cells in vitro exerted a strong dose-dependent influence on cell spreading. Specifically, plasmatocytes increased in length from about 38 μm with 2.5% CM to a maximum of about 54 μm at 100% CM; and (iv) the retarding influence of dexamethasone (an eicosanoid biosynthesis inhibitor) on cell spreading was reversed by arachidonic acid, prostaglandin H_2, and CM. Taken together, these findings indicate that both bacterial infection and eicosanoids influence hemocyte-spreading processes.

Introduction

Insect innate immune reactions to bacterial infection are registered in two broad expressions, humoral and cellular immunity. Humoral reactions involve induced biosynthesis of antimicrobial peptides, usually detected in hemolymph some hours after infection (Hoffman, 2003). Cellular reactions take place in circulating hemolymph, where circulating hemocytes directly contact bacterial cells (Gillespie et al., 1997). Cellular reactions, including phagocytosis, microaggregation, and nodulation, are launched immediately after an infection is detected. The nodulation reaction clears most infecting bacterial cells from hemolymph circulation within the first 2 h after infection (Dunn & Drake, 1983).

In the classical view, nodulation is seen as a complex process involving many steps, including attachment of granulocytes to infecting bacterial cells, degranulation of granulocytes, attraction of plasmatocytes (PLs) to the growing nodule, and spreading of PLs around the nodule (Rowley & Ratcliffe, 1981). More recently, Dean et al. (2004) proposed an alternative model of nodulation that involves the action of a novel hemocyte form, which they named hyperphagocytic (HP) cells. Under their model, the novel HPs are capable of attaching large numbers of bacterial cells, which become nuclei for an ensuing sequence of cell actions that result in formation of mature nodules.

These nodulation models draw attention to the signaling systems responsible for mediating and coordinating cellular steps in the process. Miller et al. (1994) hypothesized that eicosanoids mediate nodulation reactions to infection. Eicosanoid structures and biosynthetic pathways are described in recent works (Stanley, 2000, 2005). The eicosanoid hypothesis has been tested in approximately 20 insect species, all of which have uniformly yielded supporting results (Stanley, 2005, 2006; Stanley & Miller, 2006).

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Hemocyte spreading is one of the crucial steps in nodulation processes (Clark et al., 1997). Mandato et al. (1997) suggested that eicosanoids mediate spreading of hemocytes prepared from the greater wax moth, *Galleria mellonella*. Miller (2005) investigated this idea in detail, reporting that eicosanoids influence the elongation, but not widening, of PLs prepared from immunologically naïve tobacco hornworms, *Manduca sexta* (L.) (Lepidoptera: Sphingidae). This report revealed a specific cell reaction to bacterial infection – spreading – which depends on eicosanoids. It is not clear, however, how hemocyte–bacterial cell interactions influence cell spreading. Because hemocytes spread over mature nodules, it may be the hemocytes comprising microaggregates or nodules rather than bacterial cells, per se, somehow signal PLs to spread. Here we report on outcomes of experiments designed to more thoroughly document the roles of eicosanoids as crucial elements in cell spreading and experiments designed to test the hypothesis that in vivo bacterial infections influence cell spreading on glass surfaces.

Materials and methods

Organisms

Eggs of the tobacco hornworm, *M. sexta*, were provided by Ms. Beverley Pagura (North Carolina State University Insectary, Raleigh, NC, USA). Larvae were reared on standard culture medium in individual cups under semi-sterile conditions developed by Dunn & Drake (1983). Hemocyte suspensions were prepared from Day 2 and Day 3, fifth instars. The bacterial species *Serratia marcescens* (ATCC 13880), *Escherichia coli* (Caroline 15-5065), *Bacillus subtilis*, and *Micrococcus luteus* (ATCC 4698) used in this study were obtained from the culture collection (Department of Biological Sciences at Northern Illinois University, DeKalb, IL, USA). Bacteria were grown overnight in nutrient broth at 37 °C. Bacterial cells were suspended in saline at midlogarithmic or stationary phase at a titer of 1.5×10^7 colony-forming units per milliliter.

Reagents

Dulbecco's phosphate-buffered saline (dPBS) was purchased from Gibco (Invitrogen Corporation, Carlsbad, CA, USA). The phospholipase A_2 (PLA_2) inhibitor, dexamethasone [(11β,16α)-9-fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione], and the fatty acid, palmitic acid [hexadecanoic acid], were purchased from Sigma Chemical Company (St. Louis, MO, USA). Arachidonic acid (AA; 5,8,11,14-eicosatetraenoic acid), the cyclooxygenase (COX) product, prostaglandin H_2 (PGH_2), and the lipoxygenase (LOX) product, 5(S)-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HPETE), were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Injections and hemolymph collection

Hornworms were anesthetized by chilling on ice for 15 min, then surface sterilized by swabbing their exteriors with 95% ethanol (EtOH). Injections were performed as described in Miller et al. (1994). Briefly, for each injection (using a Hamilton 701 microsyringe from Hamilton, Reno, NV, USA), a syringe needle was inserted into the intersegmental suture between and just above the last two spiracles, then moved forward into the immediate anterior segment, keeping the needle parallel to the body wall to avoid injuring the alimentary canal. The plunger was then depressed, and the needle withdrawn with care to ensure that hemolymph did not leak out of the insect.

Control larvae were injected with 10 µl EtOH. Test larvae were injected with selected pharmaceuticals, all dissolved in 10 µl EtOH. Experimental larvae were treated with the PLA_2 inhibitor dexamethasone (dex; 10 µg per larva). For rescue experiments, the standard dex treatment was followed by an additional injection of AA (3.0 µg per larva), palmitic acid (2.5 µg per larva), PGH_2 (2.5 µg per larva), or 5-HPETE (2.5 µg per larva).

Bacterial species, suspended in saline, were injected in 100 µl aliquots (approximately 10^6 bacterial cells), using a sterile 26-gauge 0.5-inch needle attached to a 1-ml syringe (BD, Franklin Lakes, NJ, USA).

Hemolymph was collected by the pericardial puncture procedure described by Horohov & Dunn (1982). Briefly, a 20-gauge sterile, siliconized needle was inserted anteriorly at the thoracic abdominal junction such that the needle penetrated into the pericardial sinus. Freely dripping hemolymph (approximately 500 µl per larva) was collected into a chilled, sterile polypropylene 1.5-ml centrifuge tube preloaded with 500 µl of cold dPBS. The hemolymph was gently mixed with dPBS by inverting the test tube several times. The hemolymph suspensions were immediately used in experiments.

Plasmatocyte-spreading assay

A hemolymph suspension was prepared as previously described. A 100 µl hemolymph suspension (approximately 2.2×10^5 cells) was applied to a clean sterile glass cover slip and allowed to settle for 7 min. Unattached hemocytes and any remaining hemolymph were then removed by gently washing the glass cover slip with four 500 µl volumes of dPBS. The glass cover slip with attached hemocytes was then placed in the bottom of a well of a sterile plastic 24-well culture plate (Corning Costar, Corning, NY, USA) and then overlaid with 500 µl fresh dPBS or an experimental treatment in dPBS. The hemocytes were allowed to spread

for 1 h at room temperature (22 °C). After the spreading time, the preparations were gently washed with four 500 µl volumes of dPBS. The hemocytes were then fixed with 500 µl of 3.7% formaldehyde in dPBS for 10 min. The preparations were washed again with four 500 µl volumes of dPBS, stained with hemo tox 3 (Fisher Scientific, Pittsburgh, PA, USA) and viewed with a Nikon E600 light microscope at 400× total magnification.

Plasmatocyte length was defined as the length of the long axis through the cell; width was defined as the short axis taken through the nucleus at a 90° angle to the long axis. Only PLs with a length greater than 15 µm were measured. It might be thought that cells that did not spread beyond 15 µm were not PLs, however, our earlier studies on microaggregation and nodulation (Miller et al., 1994; Miller & Stanley, 2001) would indicate that PLs are not substantially depleted from circulation at 15 min post-injection (PI) and our hemolymph samples contain a fair representative mixture of all hemocyte types. Quantification of differences arising from treatments was performed by measuring the lengths and widths of 50 PL per treatment for each of 3–12 replicates (i.e., a total of 150–600 cells were measured per treatment).

Digital images were taken with a Nikon digital camera mounted to the light microscope. Estimates of PL length and width were made using ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/, 1997–2006).

Background experiments

To control for the possibility that saline (the media for bacterial suspensions) might influence hemocyte spreading, a series of background control experiments was conducted to determine the level of background spreading of PLs from the tobacco hornworm, *M. sexta*. Hemocytes were prepared for assay from untreated control larvae as previously described. Experimental larvae were anesthetized on ice and injected with 100 µl of saline and allowed to incubate for 30 min PI. Hemolymph was collected by the pericardial puncture procedure and prepared for assay as previously described. The degree of PL spreading was then assessed as described.

The influence of different bacterial species on cell spreading

Control larvae were injected with 100 µl of saline and received no further treatment. Experimental larvae were injected with 100 µl bacterial suspension prepared from *S. marcescens*, *B. subtilis*, *E. coli*, or *M. luteus* and incubated for various times as described in Results. After incubation, hemolymph from each group was collected by the pericardial puncture procedure and prepared for assay as previously described. The degree of PL spreading was then assessed as described.

The influence of species-specific conditioned medium on cell spreading

Conditioned medium (CM) was prepared as described in Miller & Stanley (2001). Briefly, hemolymph was collected by the pericardial puncture procedure in a sterile polypropylene 1.5-ml centrifuge test tube preloaded with 500 µl cold dPBS. The hemocyte titer was adjusted to approximately 2.2×10^6 hemocytes per milliliter of suspension. One milliliter of hemolymph suspension was then challenged by adding 100 µl of bacterial suspension. The preparation was gently mixed by inverting the test tube several times and then incubated for 1 h at 26 °C in an environmental shaker at 100 r.p.m. After the incubation period, the preparation was filtered through a 0.22-µm filter (Millipore Corporation, Billerica, MA, USA). The resulting filtrate, CM, was then placed on ice for immediate use.

Hemolymph used in the preparation of CM came from untreated larvae. Control CM was prepared by exposing 1 ml hemocyte suspensions to 100 µl saline. Experimental CM was prepared by exposing 1 ml hemolymph suspensions to 100 µl bacterial suspensions (either *S. marcescens*, *B. subtilis*, *E. coli*, or *M. luteus*) for either 1 h (1 h-CM) or 3 h (3 h-CM). Conditioned medium was then prepared by filtration as previously described. These CM preparations were then overlaid on untreated hemocytes prepared for assay as previously described. Plasmatocyte spreading was assessed as described.

To control for the possibility that filterable bacterial modulants may confound interpretation of the results of experiments with CM, we prepared CM in the presence of only bacterial cells, following the procedure just described. Media conditioned for 1 h in the presence of bacteria was used in control experiments as shown in Results.

The influence of conditioned media dosages on cell spreading

Media conditioned for 1 h in the presence of bacteria was prepared as just described. The resulting CM preparations were then diluted with dPBS to 2.5, 5, 12.5, 25, 50, and 100% CM. These six CM concentrations were overlaid on untreated hemocytes prepared for assay. After standard incubation periods, PL spreading was assessed.

Rescue experiments

Control larvae were treated with 10 µl EtOH and allowed to incubate for 30 min as previously described. Hemolymph suspensions were prepared for assay and PL spreading was assessed as previously described. Experimental larvae were first injected with 10 µl dex and allowed to incubate for 30 min. Dexamethasone-treated larvae were divided into seven subgroups. The first dex-treated subgroup received no further treatment. The second subgroup received an additional injection of 10 µl EtOH.

The third subgroup received an additional injection of 10 µl AA, which is substrate for eicosanoid biosynthesis. The fourth subgroup received an additional injection of 10 µl palmitic acid, which is not a substrate for eicosanoid biosynthesis. The fifth subgroup received an additional injection of 10 µl PGH_2. The sixth subgroup received an additional injection of 10 µl 5-HPETE. Hemolymph preparations were prepared for assay and PL spreading was assessed as previously described. The seventh subgroup did not receive additional treatment. However, conditioned medium prepared with the bacterium, *S. marcescens*, was overlaid on dex-treated hemocytes adhered to cover glass. Plasmatocyte spreading was then assessed as previously described.

Statistical analyses

Significant treatment effects were confirmed by analysis of variance in the general linear models procedure at $P<0.05$. Where appropriate, significant differences among treatment means were determined by protected least significant differences (LSD) test (SAS Institute, Inc., Cary, NC, USA).

Results

Background control experiments reveal typical cell spreading recorded in PLs prepared from untreated larvae and from saline-injected larvae. The average length of PLs from untreated larvae was about 36 µm with a corresponding width of about 16 µm. Similarly, PLs from larvae treated with saline elongated to an average length of 39 µm (not different from untreated controls) with a corresponding width of about 11 µm (significantly different from untreated controls).

The influence of bacterial challenge on PL spreading

As can be seen in Figure 1, bacterial challenge exerted a profound influence on cell spreading. At 15 min PI, PLs from control larvae averaged about 33 µm in length and about 17 µm in width (Figure 1A). Hemocytes from hornworms infected with *S. marcescens*, *E. coli*, *M. luteus*, or *B. subtilis*, however, were considerably different. The hemocytes appear round and none of the hemocytes were greater than 15 microns in any dimension (Figure 1B). The situation was the same at 60 min PI, with the exception that PLs prepared from hornworms infected with *S. marcescens* elongated to an average length of 23 µm.

By 180 min PI, the influence of bacterial challenges on PL elongation had faded. Plasmatocytes prepared from hornworms infected with *M. luteus*, *E. coli*, or *B. subtilis* averaged about 40 µm in length, significantly longer than controls. Plasmatocytes prepared from hornworms infected with *S. marcescens* were a little shorter at about 25 µm.

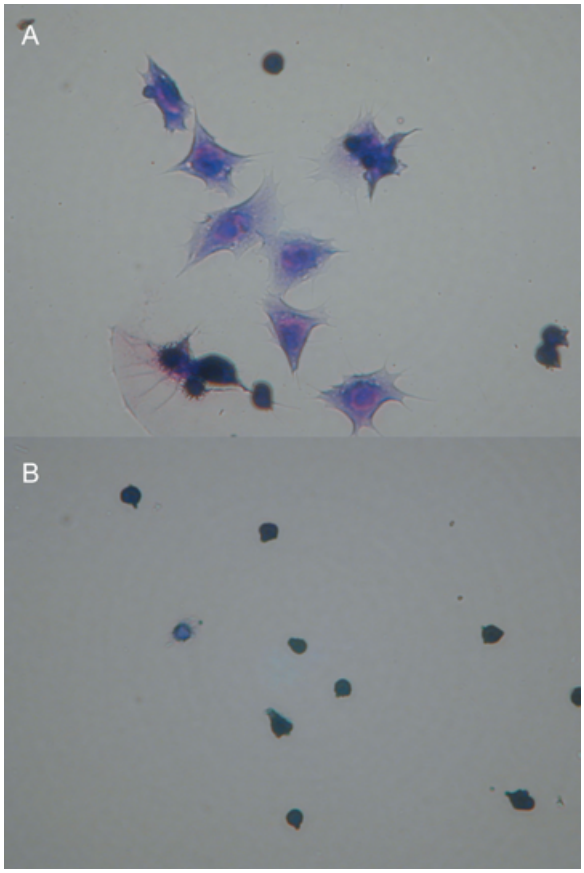

Figure 1 Photomicrographs of hemocytes prepared from (A) control and (B) bacterial-injected tobacco hornworms, *Manduca sexta* at 1 h post-treatment.

The influence of conditioned medium on plasmatocyte spreading

The influence of media conditioned by exposure to each of four bacterial species on PL spreading is displayed in Table 1. The PLs-treated saline controls elongated to a mean length of about 39 µm for 1 h treatments and about 36 µm for 3 h treatments.

Elongation reactions of PLs treated with 1 h-CM were fairly similar to controls, exactly so for CM prepared with *S. marcescens*. Plasmatocytes treated with 1 h-CM prepared with *E. coli* and *M. luteus* were slightly, but significantly longer than control-treated PLs at about 44 and 43 µm. Plasmatocytes treated with 1 h-CM prepared with *B. subtilis* were slightly, again significantly, shorter than controls at about 35 µm. The situation was different for PLs treated with 3 h-CM. Plasmatocytes treated with 3 h-CM prepared from *S. marcescens*, *E. coli*, and *B. subtilis* elongated to about 50 µm, while PLs treated with 3 h-CM prepared from *M. luteus* were even longer at approximately 55 µm.

Table 1 The influence of species-specific conditioned medium (CM) prepared at 1 and 3 h on plasmatocyte elongation. Conditioned medium was prepared by exposing 1 ml hemolymph suspensions to 100 µl of saline or bacterial suspensions (either *Serratia marcescens*, *Bacillus subtilis*, *Escherichia coli*, or *Micrococcus luteus*) for either 1 (1 h-CM) or 3 h (3 h-CM); it was then prepared by filtration as described. Each CM preparation was then overlaid on untreated hemocytes prepared for assay as described. Plasmatocyte spreading was assessed as described. Values represent the mean lengths and widths (microns ± SEM) of hemocytes after spreading on a glass cover slip for 60 min

	1 h-CM[1]		3 h-CM[1]	
Treatment	Length (± SEM)	Width (± SEM)	Length (± SEM)	Width (± SEM)
Saline control (n = 12)	38.7 (± 0.13)a	12.2 (± 0.75)d	36.3 (± 0.28)a	16.6 (± 1.16)e
Serratia marcescens (n = 5)	38.2 (± 0.34)a	13.7 (± 0.29)d	50.1 (± 0.18)b	17.0 (± 0.18)e
Escherichia coli (n = 5)	43.7 (± 0.26)b	15.7 (± 0.23)e	50.8 (± 0.18)b	17.1 (± 0.09)e
Bacillus subtilis (n = 5)	34.7 (± 0.30)c	13.5 (± 0.14)d	49.7 (± 0.18)b	15.1 (± 0.11)e
Micrococcus luteus (n = 5)	42.6 (± 0.19)b	16.0 (± 0.28)e	54.6 (± 0.17)b	13.5 (± 0.11)d

[1]Values followed by the same letter are not significantly different from each other (LSD, $P<0.05$).

The influence of conditioned medium dosage on plasmatocyte spreading

The influence of 1 h-CM dosage on PL elongation is displayed in Figure 2. Control CM yielded PLs with an average length of about 39 µm. Using media conditioned with *S. marcescens*, we recorded increasing PL lengths with increasing CM concentrations, from about 38 µm with 2.5% CM to a maximum of about 54 µm at 100% CM. We registered roughly similar patterns from dosage experiments with *M. luteus*, *E. coli*, and *B. subtilis*.

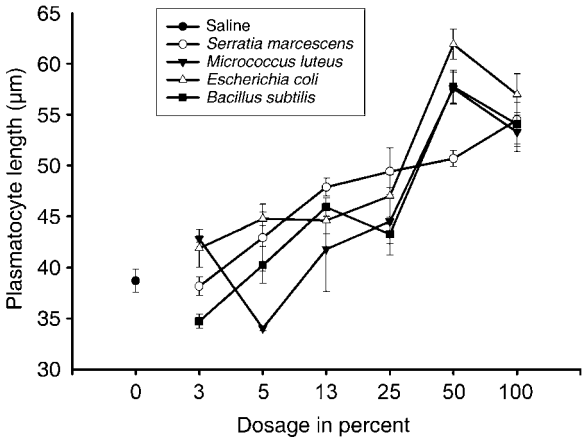

Figure 2 The influence of conditioned medium (CM) dosage on elongation of plasmatocytes prepared from untreated tobacco hornworms. Conditioned medium was prepared from each of the indicated bacterial species and overlaid onto plasmatocytes as described in Materials and methods. Data points represent the means of at least four independent replicates and the error bars represent 1 SEM.

Rescue experiments

The data presented in Figure 3 show that the retarding influence of dex on PL spreading was reversed with the addition of AA, PGH_2, or CM prepared from *S. marcescens*. Control PLs elongated to an average length of about 42 µm and an average width of about 11 µm. Plasmatocytes treated with dex or dex plus either ethanol, palmitic acid, or 5-HPETE were significantly shorter (at roughly 30 µm) than PLs treated with dex plus either aracidonic acid, PGH_2, or 1 h-CM prepared from *S. marcescens* (at >40 µm).

We considered the possibility that medium conditioned in the presence of bacterial cells and hemocytes could be contaminated by the presence of bacterial modulants that passed through the filter step. We controlled for this possibility by preparing CM using only bacterial cells of each species. The data in Table 2 show that media conditioned in the presence of solely bacterial cells did not influence the elongation of control hemocyte preparations and did not reverse the influence of dex on PL elongation.

Discussion

In this article, we report on the influence of eicosanoids and of in vivo bacterial challenge on elongation behavior of hemocytes prepared from tobacco hornworms, *M. sexta*. Cell spreading is an integral part of the nodulation reaction to bacterial infections (Lavine & Strand, 2002). The outcomes of our protocols support our hypotheses that eicosanoids act in cell spreading and that bacterial challenge influences cell spreading, as summarized here: (i) hemocytes prepared from challenged hornworms at 15 and 60 min PI were fundamentally altered in size (all less than the 15 µm counting cut-off) and none of the hemocytes exhibited cell-spreading behavior; (ii) the

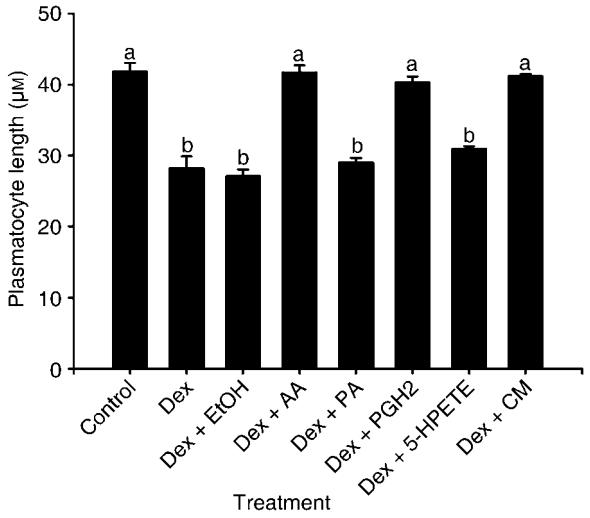

Figure 3 Arachidonic acid, PGH_2, and conditioned medium (CM) reversal of the retarding influence of dex on plasmatocyte elongation. Larvae were treated with ethanol (control, n = 10), dex (dex, n = 5), dexamethasone and EtOH (dex + EtOH, n = 8), dexamethasone and AA (dex + AA, n = 8), dexamethasone and palmitic acid (dex + PA, n = 5), dexamethasone and PGH_2 (dex + PGH2, n = 8), dexamethasone and 5-HPETE (dex + 5-HPETE, n = 4), and conditioned media overlaid on hemocytes pretreated with dexamethasone (dex + CM, n = 8). Each bar (+ SEM) represents the mean length (μm) measured after spreading on a glass cover slip for 60 min. Bars capped with the same letter are not significantly different (LSD, $P<0.05$). The corresponding widths for each group were not significantly different except for results with CM (data not shown).

influence of bacterial challenge on cell spreading declined with time; (iii) CM exerted a strong dose-dependent influence on cell spreading; (iv) the retarding influence of dex on cell spreading was reversed by AA, PGH_2, and by CM; and (v), CM prepared with only bacterial cells did not influence cell elongation. Taken together, these findings indicate that bacterial infection and eicosanoids are involved in hemocyte-spreading processes.

Because cell spreading is an important aspect of hemocyte biology, it is not surprising that spreading is influenced by a still unknown number of endogenous factors. Clark et al. (1997) identified a 23-amino acid PL-spreading peptide that induces PLs from the moth *Pseudoplusia includens* to spread on foreign surfaces, including glass slides. The peptide exerts its influence on cell spreading by binding to a specific cell surface receptor (Clark et al., 2004). We can expect other peptidyl compounds to act in cell spreading because the PL-spreading peptide influences a subpopulation of PLs rather than all of them (Clark et al., 1997).

Aside from peptidyl factors, eicosanoids influence PL elongation – but not widening – on glass slides. Miller (2005) reported significantly reduced elongation in hemocytes prepared from hornworms treated with a range of eicosanoid biosynthesis inhibitors. The limiting influence of dex, an inhibitor of the first step in eicosanoid biosynthesis, PLA_2, was reversed by treating hornworms with AA, the common precursor of eicosanoid biosynthesis. Here we add new information on eicosanoid actions in hemocyte spreading. Insects express two major pathways of eicosanoid biosynthesis (Stanley, 2006). The COX pathway is responsible for producing prostaglandins (PG). There are several LOX pathways, which yield a wide assortment of LOX products. The Miller (2005) report indicates that two LOX inhibitors, esculetin and caffaic acid, reduced cell spreading. Here we note that the LOX product 5-HPETE did not reverse the dex influence on hemocyte elongation. We interpret this to mean that although LOX products are important in cell elongation, neither 5-HPETE, nor its immediate metabolite 5-hydroxyeicosatetraenoic acid, is among them. We note that in addition to AA, PGH_2, the root PG from which other primary PGs are formed by enzymatic action, reversed the influence of dex

Table 2 The influence on cell spreading of media conditioned in the presence of bacterial species without hemocytes. Conditioned medium (CM) was prepared in the presence of each of the indicated bacterial species. As described in the Materials and methods section, in separate experiments, medium conditioned with each bacterial species was overlaid on hemocytes and spreading was assessed following standard protocols. The values represent the mean lengths and widths (microns ± SEM; n = 3) of hemocytes after spreading on a glass cover slip for 60 min

	Untreated hemocytes[1]		Dex-treated hemocytes[1]	
Treatment	Length (± SEM)	Width (± SEM)	Length (± SEM)	Width (± SEM)
Serratia marcescens	39.0 (± 3.2)a	11.7 (± 0.92)e	33.7 (± 1.6)c	12.8 (± 0.5)g
Escherichia coli	43.5 (± 2.8)b	10.8 (± 0.8)e	36.2 (± 3.0)d	12.0 (± 0.3)g
Bacillus subtilis	45.2 (± 4.3)b	9.5 (± 0.3)f	35.6 (± 1.5)d	9.4 (± 0.4)f
Micrococcus luteus	46.2 (± 3.0)b	9.6 (± 0.7)f	35.2 (± 1.4)d	9.8 (± 1.0)f

[1]Values followed by the same letter are not significantly different from each other (LSD, $P<0.05$).

on cell spreading. There are two PGH_2 action mechanisms in mammals (Stanley, 2000). First, as just noted, PGH_2 is substrate for enzymatic conversion to PGE_2, PGD_2, PGF_2, PGI_2, and thromboxane. These compounds exert their actions via specific cell surface G-protein coupled receptors. Second, PGH_2 itself is a biologically active compound, acting through a specific PGH/thromboxane receptor. We propose that either PGH_2 or its PG metabolites act in cell spreading.

The point that eicosanoids act in cell spreading is bolstered by our finding that CM reversed the influence of dex on cell spreading, as seen in earlier work (Miller & Stanley, 2001). With respect to cell elongation, the CM-mediated reversal was registered in a dose-dependent pattern. The dose–response relationship strongly supports the idea that CM operates in a physiological way, most likely through eicosanoid–receptor interactions. In another experiment we considered the influence of conditioning time on the ability of CM to stimulate cell elongation. Conditioned medium was prepared by exposing hemocytes to bacterial cells for either 1 or 3 h. Hemocytes from untreated hornworms were overlaid with either 1 h-CM or 3 h-CM. Relative to saline control hemocytes, cells treated with 1 h-CM (by exposure to *M. luteus* or *E. coli*) were significantly longer. Treating hemocytes with 3 h-CM resulted in greater increases in cell length. This was true for all four bacterial species, from which it can be inferred that 3 h-CM contained higher concentrations of cell elongation-mediating compounds. The idea that three separate hemocyte treatments (AA, PGH_2, and CM) reversed the effects of dex forges a strong linkage between bacterial infection and eicosanoid actions in cell spreading.

A potential flaw in the logic of these experiments lies in the possibility that filterable bacterial modulants may contaminate CM and these filterable molecules may be responsible for the effects ascribed to hemocyte-produced eicosanoids. We controlled for this possibility by creating CM in the presence of solely bacterial cells. Media prepared in this way exerted no influence on control hemocyte preparations and did not reverse the influence of dex on cell spreading. These results support our view that products of the hemocytes, not bacterial cells, are responsible for the influence of CM on cell actions.

We found that bacterial infections exert a profound and biphasic influence on PL spreading on glass surfaces (Figure 1). In the early phase of infection, during which many free bacterial cells circulate in the hemolymph, the infection appears to influence cell size and to disable spreading behavior. We do not know whether the bacterial cells somehow actively inhibit one or more of the cellular dynamics involved in spreading or whether biochemical factors necessary to activate spreading are not yet present in hemolymph. In either case, hemocytes prepared from tobacco hornworms soon after infection did not appear normal and they did not exhibit spreading behavior. For hemocytes harvested during the later phase of infection, however, when nodulation processes have severely depleted the numbers of free bacterial cells (as well as hemocytes) from circulation, we registered no bacterial influence on cell spreading. This is congruent with the apparent kinetics of nodule formation following infection. Using in vitro hemocyte preparations, we recorded bacterial-stimulated increases in microaggregation reactions at 15 min PI (Miller & Stanley, 2001). Lipopolysaccharide challenge stimulated increased microaggregate formation as early as 5 min PI (Miller & Stanley, 2004). Cellular defense reactions begin with microaggregation, which can be recorded earlier than nodulation. Cell spreading is thought to be one of the final steps in nodulation because a layer of PLs spread over mature nodules, after which the melanization process is launched. These comments, however, leave open important questions about the mechanisms of bacterial influences on the shape and spreading of PLs early in the infection cycle.

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